# The Chemistry of Allergens

The castor bean allergenic fraction, CB-1A, is one of a class of substances designated as natural proteoses, or 1A fractions, isolated from oilseeds and nuts by a general procedure known as the CS-1A (cottonseed) procedure (1). Those seeds and nuts yielding typical 1A fractions were: almond nuts, brazil nuts, castor beans, cottonseed, filbert nuts, flaxseed, kapok seed, and mustard seed (1). It was recognized that CB-1A and the other 1A fractions contained the principal allergenic specificities and were immunologically distinct from other allergens and antigens present in their respective source materials. The 1A fractions are complex mixtures of low molecular weight proteins and polysaccharidic proteins. Although the 1A fractions as well as the other antigens from the various oilseeds are immunologically distinct from each other, the principal components of each 1A mixture were regarded as having a common antigenic and allergenic specificity (1–6). However, the presence in Fractions CS-1A and CB-1A of other antigens with different specificities was recognized (4, 7, 8). It has been shown that the polysaccharidic portions of the molecules of Fraction CS-1A from cottonseed and Fraction CB-1A enhanced their sensitizing capacities in guinea pigs, but did not alter their specificities or their shocking or precipitinogenic capacities when compared on a nitrogen basis (9, 10).

By means of an ion exchange fractionation, Layton, DeEds, and Moss (11) found that CB-1A contained 12 major protein species. Later, Layton, Moss, and DeEds (12) fractionated CB-1A by DEAE-cellulose chromatography and tested the fractions for antigenic specificity by the Schultz-Dale technique. Antigenic differences between the fractions were interpreted to indicate the possible existence of several specific antigenic or allergenic components. Layton *et al.* (13) then described a paper electrophoresis fractionation of CB-1A and reported that all of the bands were antigenic as determined by passive cutaneous anaphylaxis in guinea pigs. No evidence was presented to show that each band exhibited a distinct antigenic specificity. By means of passive transfer tests on monkeys with the use of human reaginic serum, Layton *et al.* (13, 14) reported that there were at least five distinct allergenic specificities present in bands separated by paper electrophoresis, but no experimental evidence was published.

The purpose of this paper is to describe: (*a*) an ion exchange fractionation of CB-1A, (*b*) the cellulose acetate electrophoretic characterization of the fractions of CB-1A, and (*c*) the antigenic specificity relationships of the fractions as determined by Schultz-Dale tests, by gel double diffusion, by immunoelectrophoresis, by a combination of the last two methods, and by antibody absorption techniques.

## EXPERIMENTAL PROCEDURE

### *Materials*

*CB-1A*—Fraction CB-1A was isolated from defatted castor beans as previously described (1, 15).

*(CB-1A)E and (CB-1A)D*—These fractions were prepared from Fraction CB-1A by dialysis in a Visking casing.[1] A sample of 62 g of Fraction CB-1A, dissolved in 620 ml of water, was dialyzed against 1500-ml portions of water, and 5 dialysate fractions were obtained during successive 24-, 68-, 168-, 168-, and 167-hour periods. The dialysate fractions contained 1.0, 2.8, 5.4, 4.3, and 3.8% of the total nitrogen, respectively. The fraction remaining inside the membrane was isolated by precipitation with 4 volumes of ethanol and designated (CB-1A)E. Fraction (CB-1A)D was isolated from the combined concentrated dialysate solution in the same manner. The yields of (CB-1A)E and (CB-1A)D were 44.4 and 7.5 g; their nitrogen contents were 16.6 and 16.9%, and their carbohydrate contents were 5.7 and 2.8%, respectively, on an air-dried basis. The sedimentation constant of Fraction (CB-1A)E was $s_{20} = 1.98$ S.[2]

*Fraction CB-65A*—This carbohydrate-free allergen was isolated from Fraction CB-1A as previously described (6).

*Resin*—Amberlite IRC-50-XE64, from Rohm and Haas Company, Philadelphia, Pennsylvania, was conditioned by the method of Hirs, Moore, and Stein (16), except that absolute ethanol was used for washing instead of acetone. The resin was adjusted to pH 5.50 and equilibrated with 0.4 N sodium acetate buffered at pH 5.50.

*Agar*—Ionagar, No. 2, from Consolidated Laboratories, Chicago Heights, Illinois, was used.

*Buffer Solution*—Barbitone acetate buffer, Consolidated Laboratories 8.8 g per liter, pH 8.6, was used for cellulose acetate electrophoresis and immunoelectrophoresis. This buffer is approximately 0.03 M in diethylbarbiturate (17).

*Fraction CB-1A Rabbit Antiserum*—Rabbits were immunized to Fraction CB-1A by a series of inoculations with CB-1A in Freund's complete adjuvant as described for the preparation of Fraction CB-13E rabbit antiserum (18).

[1] The use of a trade name, distributor, or manufacturer is for identification only and implies no endorsement of the product or its manufacturer.

[2] The authors are indebted to Dr. Robert H. Jackson for this determination.

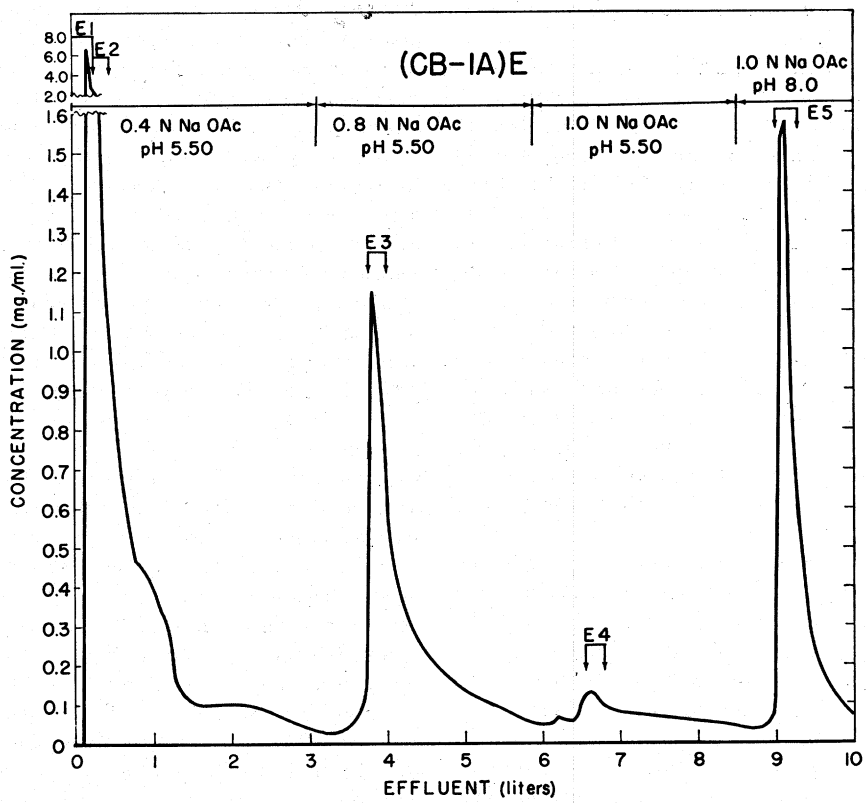


FIG. 1. Ion exchange fractionation of E on Amberlite IRC-50 at pH 5.50; pH of Fraction E5 effluent ranged from 5.48 to 5.63

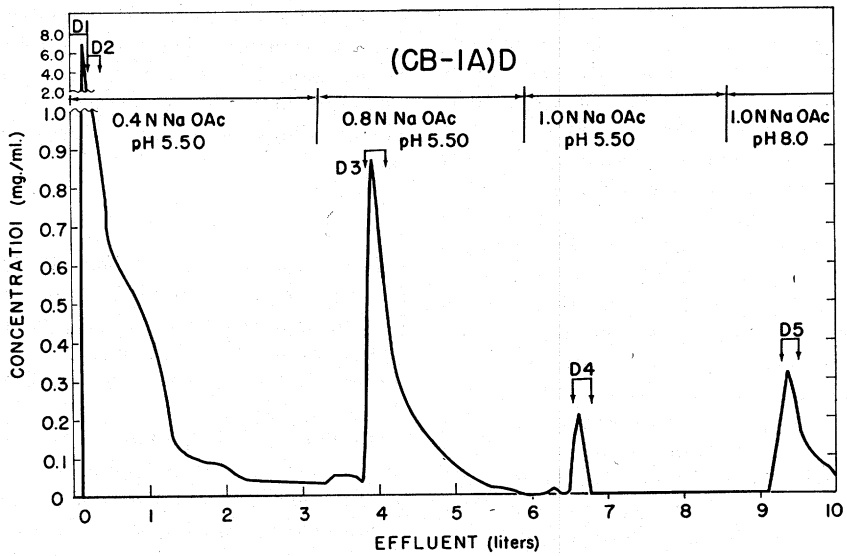


FIG. 2. Ion exchange fractionation of D on Amberlite IRC-50 at pH 5.50; pH of Fraction D5 effluent ranged from 5.53 to 5.67

## *Methods*

*Ion Exchange Fractionation of E*[3] *and D*—The column was a glass tube 20 mm inside diameter and 735 mm long with a coarse glass filter sealed in the lower end. The height of the resin was 665 mm. A sample of 3 g of Fractions E or D, dissolved in 25 ml of 0.4 N sodium acetate buffered at pH 5.50, was introduced into the column. Elution was done, successively, with 0.4, 0.8, and 1.0 N sodium acetate solutions buffered at pH 5.50, and finally with 1.0 N sodium acetate solution at about pH 8. The absorption of the effluent was monitored at 280 mμ. Fractions were isolated from the effluent as indicated in Figs. 1 and 2, by precipitation with 4 volumes of ethanol at 5°. The precipitate was separated by centrifuging, washed once with 50 ml of cold ethanol, dried in a vacuum over calcium chloride, and then exposed to air. A negligible amount of material remaining on

[3] The following abbreviations are used: 1A, CB-1A; E, (CB-1A)E; D, (CB-1A)D; E1, E2, E3, E4, and E5, ion exchange fractions from (CB-1A)E; D1, D2, D3, D4, and D5, ion exchange fractions from (CB-1A)D; 65A, the carbohydrate-free allergenic fraction, CB-65A; antiserum, CB-1A rabbit antiserum.

the column after elution with 1.0 N sodium acetate was removed with dilute ammonium hydroxide.

*Cellulose Acetate Electrophoresis*—The Shandon Electrophoresis apparatus and 5- × 17-cm strips of Oxoid cellulose acetate, both from Consolidated Laboratories, (19) were used. A sample of 12 μl of a solution containing 2.0 mg of fraction nitrogen per ml in barbitone buffer was placed in the center of the strip. Electrophoresis was conducted at 22–25° at a constant current of 2 ma for 4 hours. The voltage was 240 at the start and 140 at the end. The strips were dried at 95° for 15 minutes. The dried strips were stained with ponceau S dye by the following procedure which is a modification devised to prevent diffusion of the bands of soluble protein before they were immobilized by reaction with the dye. A solution of 250 ml of 0.2% ponceau S in 3% trichloroacetic acid was diluted with 750 ml of absolute ethanol. The strip was quickly lowered into a cylinder containing the stain, and left for 30 minutes. The excess stain was removed by repeated washing in 5% acetic acid. The strips were dried at room temperature.

*Schultz-Dale Technique*—Virgin female guinea pigs, weighing about 250 g, were sensitized with a single subcutaneous injection of alum-precipitated fraction containing 1 mg of solid. The incubation period ranged from 26 to 57 days. Challenge doses were administered in terms of protein nitrogen content. The Schultz-Dale experiments were conducted by techniques previously described (20).

*Gel Double Diffusion Technique*—The Ouchterlony technique (21, 22) was used. Gel diffusion patterns were formed in solidified 0.5% agar with Feinberg cutters (23). Patterns are designated as follows. Pattern 1 had a central well circled by 6 concentric outer wells. Pattern 2 had 18 wells arranged in two rings of 6 and 12. Pattern 3 had 4 wells in a lozenge pattern. Volumes of solutions used in the wells were: Pattern 1, 0.09 ml in outer wells and 0.14 ml in inner wells; Pattern 2, 0.03 ml; and Pattern 3, 0.09 ml. The agar and fraction solutions were made up in buffered 0.87% sodium chloride solution, pH 7.0, containing 0.01% Merthiolate. A single filling of antiserum was added 24 hours before a single filling of fraction solution because of the rapid diffusion of the fractions. The diffusion patterns were allowed to develop at 24 ± 1° for 5 or 6 days.

*Absorption Technique*—The amount of each fraction required to absorb from antiserum all antibodies specific for antigens contained in the fraction was determined quantitatively. Concentrations ranging from 0.010 to 0.105 mg of fraction nitrogen per ml of antiserum in 0.005-mg increments were used. Calculated volumes of a water solution containing 0.25 mg of fraction nitrogen per ml were added to a series of 6-ml bottles, and the water was evaporated to dryness in a vacuum over calcium chloride. Antiserum in 1-ml amounts was added to each bottle, and the contents were swirled to dissolve the fraction. The stoppered bottles were incubated at 37° for 30 minutes, and then stored at 4° for 48 hours. The precipitate was removed by centrifuging at 1000 × *g* for 30 minutes in 15-ml stoppered tubes at 4°. Antibody excess in the supernatant fluid was determined by the gel double diffusion technique with use of Pattern 1 as follows. The absorbed antisera were placed in the outer wells, and 24 hours later a solution, containing 0.013 mg of nitrogen per ml of the fraction used for absorption, was added to the central well. The first concentration of fraction that yielded no visible precipitate after 5 days was designated "antibody neutralization point." Antigen excess in the supernatant fluid was determined similarly except that unabsorbed antiserum was placed in the central well, and after 24 hours the absorbed antisera were placed in the outer wells. The first concentration of fraction in the absorbed antisera that gave no line of precipitate after 5 days was designated "fraction neutralization point."

*Reaction of Other Fractions with Absorbed Antisera*—Antibodies to the other fractions remaining in the absorbed antisera were determined qualitatively. To insure complete removal of fraction antibodies, the antiserum was absorbed with a concentration of 0.005 mg of nitrogen per ml of antiserum in excess of the antibody neutralization point. The concentrations of fraction nitrogen per ml of antiserum used for these absorptions were: Fraction D3, 0.025; Fraction D4, 0.035; Fraction D5, 0.075; Fraction E1, 0.100; Fraction E3, 0.045; and Fraction E5, 0.060. Unabsorbed antibodies specific for antigens in the other fractions were determined by the gel double diffusion method with Pattern 3. The well indicated by a notch at the top was designated Well 1 and the other wells were numbered consecutively in clockwise direction. Unabsorbed antiserum was placed in Well 1 and absorbed antiserum in Well 3. After 24 hours, the absorbing fraction containing 0.013 mg of nitrogen per ml was placed in Well 2. Likewise, the test fraction containing 0.013 mg of nitrogen per ml was placed in Well 4. Thus, the lines of precipitate between Wells 1 and 2 show the reaction of absorbing fraction with unabsorbed antiserum (positive control). The space between Wells 2 and 3 is a negative control showing that absorption of antibodies by the absorbing fraction was complete. Any lines of precipitate between Wells 3 and 4 result from reaction between the test fraction and antibodies not removed by the absorbing fraction. Lines between Wells 4 and 1 show the reaction of the test fraction with unabsorbed antiserum (positive control). The lines of precipitate that appear between Wells 1 and 3, with some fractions, are due to the reaction of antiserum in Well 1 with excess absorbing fraction in Well 3. Final readings and photographs were taken after 5 days of diffusion.

## RESULTS

*Ion Exchange Fractionation*—Results of the ion exchange fractionation of Fractions E and D are shown in Figs. 1 and 2. Elution with each solution was prolonged to insure as complete separation of the fractions as possible. The yield, nitrogen content, and estimated carbohydrate content of the fractions are shown in Table I.

*Cellulose Acetate Electrophoresis*—Comparative results of the cellulose acetate electrophoresis of the fractions are shown in Fig. 3. Fractions E1 and D5, which represented the extremes of separation by a combination of dialysis and ion exchange fractionation, were separated from each other completely as shown by this criterion. Fraction D3 was separated from Fraction E1, except for the one of the four bands of Fraction D3 nearest to the anode. It is unlikely that a band represents a homogeneous substance, but probably contains a mixture of many very closely related substances.

*Schultz-Dale Tests*—Schultz-Dale tests were conducted with Fraction E and with preliminary fractions approximating E1 and E5. In preliminary tests, Fraction E1 did not desensitize homologously sensitized uterine muscle to subsequent test with Fraction E5 when the two were tested at the same dosage level (nitrogen basis). This observation suggested that Fraction E5

might be a stronger antigen than Fraction E1. These two fractions, therefore, were compared by the Schultz-Dale quantitative method (20). Fraction E5 was found to be 5 times more potent in eliciting anaphylactic contractions in uterine muscles irrespective of whether the animals had been sensitized with Fractions E, E1, or E5. Accordingly, in a qualitative comparison, Fraction E1 was tested at a dosage level of 50 μg of nitrogen and Fraction E5 at a dosage level of 10 μg of nitrogen. The results indicated that Fractions E1 and E5 contained antigens with identical or closely similar specificities, and that Fraction E5 contained at least one antigen not contained in Fraction E1. Because of the indicated complexity of these two fractions, it was concluded that the Schultz-Dale method was not suitable for resolving the antigenic specificity relationships of the components of Fraction 1A.

*Precipitinogenic Potencies*—The relative precipitinogenic potencies of the fractions are shown in Table II, and the method is illustrated in Fig. 4 with 1A as an example. There were two principal lines of precipitate in the fractions designated inner and outer.

TABLE I

*Ion exchange fractions from 3.0 g each of Fractions D and E*

| Fraction | Yield | | Nitrogen* | Estimated carbohydrate† |
|---|---|---|---|---|
| | *mg* | %* | % | % |
| D1 | 204 | 6.8 | 13.7 | 21.2 |
| D2 | 100 | 3.3 | 16.9 | 2.9 |
| D3 | 197 | 6.6 | 16.9 | 2.9 |
| D4 | 29 | 1.0 | 17.5 | 0.0 |
| D5 | 29 | 1.0 | 17.2 | 1.1 |
| E1 | 441 | 14.7 | 12.8 | 26.3 |
| E2 | 248 | 8.3 | 16.8 | 3.4 |
| E3 | 206 | 6.9 | 17.2 | 1.1 |
| E4 | 9 | 0.3 | 16.8 | 3.4 |
| E5 | 217 | 7.2 | 17.2 | 1.1 |

* Air-dried basis.

† Because of the limited amounts of these fractions available, carbohydrate was estimated from the nitrogen contents on the assumption that the carbohydrate-free fraction, 65A, contained 17.4% nitrogen, air-dried basis.

TABLE II

*Relative precipitinogenic potencies of ion exchange fractions of 1A**

| Fraction | Lowest concentration giving visible precipitate | | Precipitinogenic potency index† | |
|---|---|---|---|---|
| | Inner line | Outer line | Inner line | Outer line |
| | *mg fraction N/ml* | | | |
| 1A | 0.0004 | 0.0008 | 31 | 16 |
| D1 | 0.0032 | 0.0125 | 4 | 1 |
| D3 | 0.0002 | 0.0125 | 63 | 1 |
| D5 | 0.0008 | 0.0004 | 16 | 31 |
| E1 | 0.0008 | 0.0032 | 16 | 4 |
| E3 | 0.0004 | 0.0125 | 31 | 1 |
| E5 | 0.0032 | 0.0004 | 4 | 31 |
| 65A | 0.0004 | 0.0016 | 31 | 8 |

* Determined by gel double diffusion with Pattern 2, illustrated in Fig. 4. Antiserum was placed in each of the inner wells and after 24 hours, 2-fold serial dilutions of fraction were placed in the outer wells. The lowest concentration (end point) which gave a visible precipitate after 6 days was observed for each of the two principal lines of precipitate for each fraction.

† The highest end point concentration was 0.0125 mg of nitrogen per ml. The precipitinogenic potency index of a line is the ratio of 0.0125 to the end point concentration of that line of precipitate.

The "precipitinogenic potency index" was devised to compare the minimum concentrations of fraction nitrogen required to produce each of the two principal lines of precipitate. As shown in Table II, the highest end point concentration for any fraction was 0.0125 mg of nitrogen per ml. Thus, the precipitinogenic potency index for a given line was the ratio of 0.0125 to the end point concentration for that line.

The ratios of the precipitinogenic potency index values of the inner to the outer lines of Fractions E3, D3, and E5 were: 31:1, 63:1, and 1:8. From this it may be estimated that the principal antigens of Fractions E3, D3, and E5 accounted for approximately 97, 98, and 88%, respectively, of the precipitating capacities of these fractions. The designation, inner and outer, refers

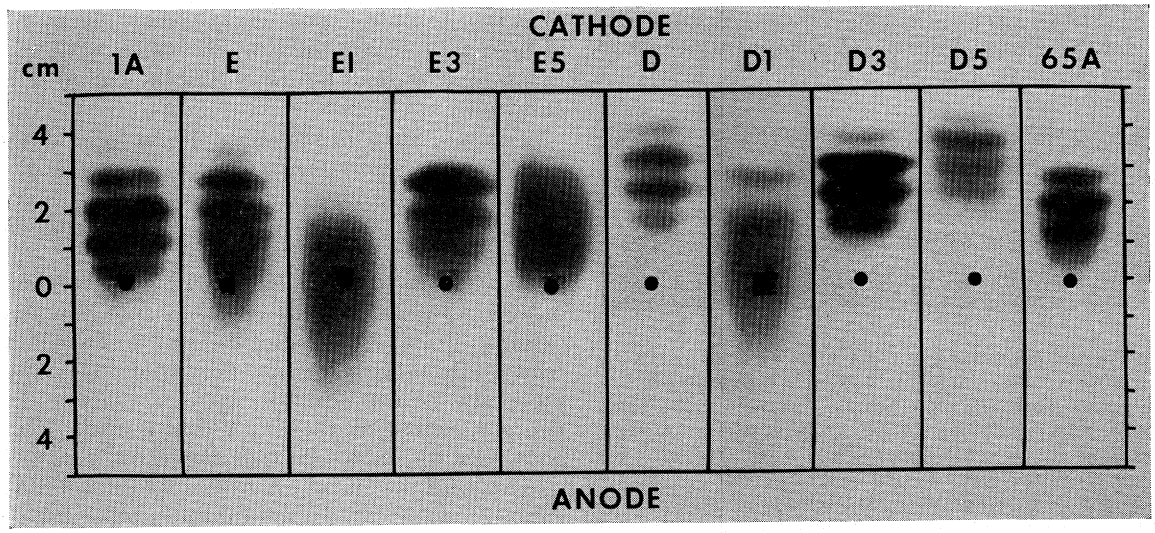


FIG. 3. Comparative cellulose acetate electrophoretograms of Fraction 1A and fractions of 1A

to the relative location of the lines on the plates and does not identify similar antigens in the various fractions. For example, the inner line of Fraction D3 joined the outer line of Fraction E5 when solutions of the two fractions containing 0.0032 mg of fraction nitrogen per ml were tested in adjacent wells (Pattern 2) with antiserum. This showed the antigenic similarity of the inner line of Fraction D3 to the outer line of Fraction E5.

*Specificity Relationships by Gel Double Diffusion*—The specificity relationships of 1A and fractions of 1A, are shown in Figs. 5 and 6. A heavy joining line of precipitate, *upper* Fig. 5, showed that an identical or closely related major specificity is common to all of the fractions.

The specificity relationships of Fraction E1 with Fractions D5 and E5 and of Fractions E1 and D3 and E3, Fig. 6, are especially pertinent to the demonstration that chemically distinct components of Fraction 1A possess a common specificity. This is particularly striking with Fractions E1 and D5 because they represent the extremes of fractionation and because their electrophoretograms indicated complete separation. A heavy joining line shows a major common specificity in these fractions. Fractions D5 and E5 have a minor common specificity not present in Fraction E1 as shown by the lower line of precipitate of Fraction D5 and of Fraction E5 which crosses the double lines of Fraction E1 and joins smoothly under the E1 well. Fractions D3 and E3 appear to have a single joining line of precipitate. Fraction E1 has a major and a minor line of precipitate both of which merge with the apparently single lines of precipitate of Fractions D5, E5, D3, and E3.

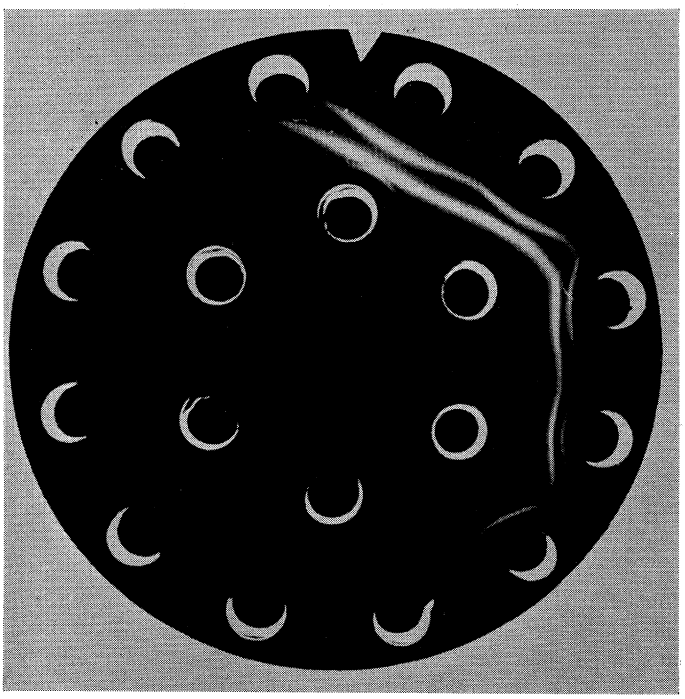

FIG. 4. Test showing the precipitinogenic potencies of components of Fraction 1A. Starting concentration was 0.025 mg of Fraction 1A nitrogen per ml in first outer well to right of notch. Two-fold serial dilutions of Fraction 1A were used in wells in clockwise direction.

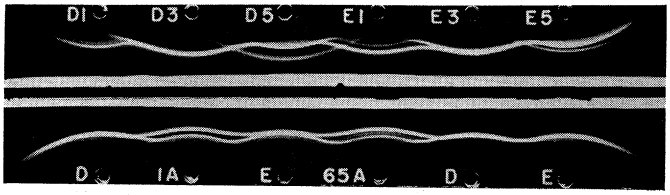


FIG. 5. Specificity relationships of 1A and fractions of 1A by gel double diffusion. Agar, 0.5% in buffered saline at pH 7.0; depth, 4 mm; glass plate, 102- × -193 mm. Antigen wells, 3 mm in diameter, 20 mm apart, and 15 mm from antiserum trough. Fraction solutions, 10 $\mu$l, containing 0.5 mg of fraction nitrogen per ml in buffered saline, pH 7.0. Antiserum trough, 3- × -150 mm, contained 2.0 ml of antiserum. Antiserum diffused 24 hours before adding fraction solutions. Immunodiffusion, 6 days at 24 ± 1°.

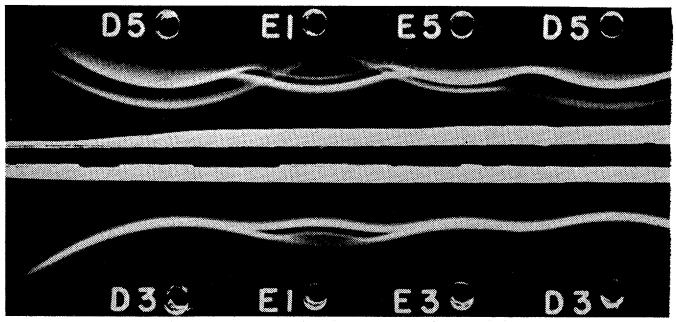


FIG. 6. Specificity relationships of Fractions D5, E1, and E5, and of Fractions D3, E1, and E3 by gel double diffusion. Conditions of experiment are the same as those described in Fig. 5, except diffusion was for 7 days.

The diffusion pattern recorded in Fig. 7 shows that precipitin lines from Fractions D3 and E3 were each resolved into two bands. Neither of these were clearly resolved in the diffusion pattern shown in the *lower part* of Fig. 6.

The capacity of Fraction D3 to produce antibodies specific for the other fractions was determined by a technique similar to that shown in Fig. 7. Solutions of Fractions D3, D4, D5, E1, E3, and E5, containing 0.025 mg of fraction nitrogen per ml, were allowed to diffuse against Fraction D3 rabbit antiserum. All of these fractions gave a heavy joining line of precipitate confirming the presence of the major common specificity. Fraction D3 had an additional faint line that probably corresponds to the faint outer line shown for Fraction D3 in Table II. Fractions D4 and E1 each gave one line in addition to the major line of precipitate. Antibodies for the minor antigenic specificity common to Fractions E5 and D5 were absent in the Fraction D3 antiserum.

A detailed study of the minor specificities indicated in some of the fractions was attempted by the gel double diffusion technique, but the resulting patterns could not be interpreted definitely. Accordingly, the problem was investigated by other techniques.

*Specificity Relationships by Absorption*—Absorption is another method of showing identity or dissimilarity of antibodies. For example, if all of the antibodies specific for one fraction are removed by antigenic precipitation (absorption), any additional precipitate produced subsequently by another fraction will indicate another specificity. A characteristic of antigen-antibody reactions of homogeneous antigens is a sharp equivalence zone, a condition in which neither excess antigen nor excess antibody appears in the supernatant solution after removal of the precipitate.

The antibody neutralization and the fraction neutralization points for the fractions are shown in Table III. The concentrations (milligram of fraction nitrogen per ml of antiserum) of the fraction neutralization and antibody neutralization points, respectively, were: Fraction D3, 0.020 to 0.020; Fraction D4, 0.020 to 0.030; Fraction D5, 0.010 to 0.070; Fraction E1, 0.030 to 0.095; Fraction E3, 0.025 to 0.040; and Fraction E5, 0.040 to 0.055. D3 was the only fraction that showed a sharp equivalence zone characteristic of antigenic homogeniety.

Specificity relationships of the fractions by the absorption technique are shown in Fig. 8, where results of testing antiserum absorbed with one fraction with the other fractions are shown. Fractions D4, D5, and E5 each completely absorbed antibodies specific for all of the other fractions as shown by the absence of lines of precipitate between Wells 3 and 4 when Fractions D4, D5, and E5 were used as absorbing antigens. All of the fractions absorbed antibodies specific for Fraction D1. Antiserum absorbed with Fractions E1, E3, and D3 retained antibodies which reacted to give a single relatively weak line of precipitate when tested with Fractions D5 and E5 as shown by the lines between Wells 3 and 4 when Fractions E1, and E3 and D3 were used as absorbing antigens. Antiserum absorbed with Fraction E1 gave two weak lines of precipitate and antiserum absorbed with Fraction D3 gave one weak line when tested with Fraction D4. The sharpness of the equivalence zone of Fraction D3 is confirmed, Fig. 8, because there is no precipitate between Wells 1 and 3 caused by antibodies in unabsorbed antiserum reacting with excess antigen in antiserum absorbed with Fraction D3.

These absorption studies confirm conclusions from the other studies that chemically distinct components of Fraction 1A have a major common or similar antigenic specificity. This conclusion is corroborated by the fact that D4, a sharply discrete fraction amounting to only 1% of Fraction D and 0.17% of Fraction 1A, completely absorbed antibodies specific for all of the separated fractions as well as for 1A.

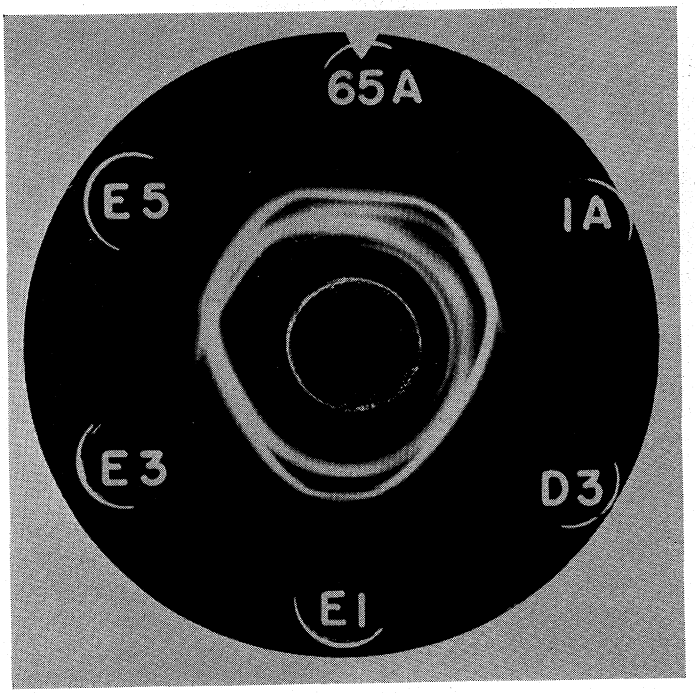


FIG. 7. Specificity relationships showing resolution of Fractions D3 and E3 into two lines of precipitate by gel double diffusion. Conditions of experiment similar to those of Fig. 5. The concentration of test solutions (0.09 ml) was 0.025 mg of fraction nitrogen per ml.

TABLE III

*Determination of fraction neutralization point and antibody neutralization point by absorption**

| Concentration of fraction N | Absorbing fraction† | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | D3 | | D4 | | D5 | | E1 | | E3 | | E5 | |
| | AG‡ | AB‡ | AG | AB | AG | AB | AG | AB | AG | AB | AG | AB |
| *mg/ml antiserum* | | | | | | | | | | | | |
| 0.005 | 0 | | 0 | + | 0 | | | | | | | |
| 0.010 | 0 | + | 0 | + | 0 | | | | | | | |
| 0.015 | 0 | + | 0 | + | + | | | | | | | |
| 0.020 | 0 | 0 | 0 | + | + | | | | 0 | | 0 | + |
| 0.025 | + | 0 | + | + | + | | 0 | | 0 | + | | |
| 0.030 | + | 0 | + | 0 | | | 0 | | + | + | 0 | + |
| 0.035 | + | 0 | | | | | + | | + | + | | |
| 0.040 | | | | | | | + | | + | 0 | 0 | + |
| 0.045 | | | | | | | | | | 0 | + | + |
| 0.050 | | | | | | | | | | 0 | + | + |
| 0.055 | | | | | | | | | | 0 | + | 0 |
| 0.060 | | | | | | + | | | | | + | 0 |
| 0.065 | | | | | | + | | | | | | |
| 0.070 | | | | | | 0 | | + | | | | |
| 0.075 | | | | | | 0 | | + | | | | |
| 0.080 | | | | | | | | + | | | | |
| 0.085 | | | | | | | | + | | | | |
| 0.090 | | | | | | | | + | | | | |
| 0.095 | | | | | | | | 0 | | | | |
| 0.100 | | | | | | | | 0 | | | | |

* Method described in "Experimental Procedure."

† 0, no precipitate; +, visible precipitate.

‡ AG, fraction neutralization point; AB, antibody neutralization point.

*Immunoelectrophoresis*—The immunoelectrophoretic patterns of 1A and the fractions of 1A are shown in Fig. 9. Fraction E1 gave a single line of precipitate. Fractions E3 and D3 gave lines of precipitate that were single in the central part but split on the ends. Fractions E5 and D5, each had a single principal line, E5 had two, and D5 had one lesser line of precipitate. Fractions E and D had two lines of precipitate and Fractions 1A and 65A had three lines.

The phenomenon of split ends of an apparently single line of precipitate from E3 and D3 was previously noted in the double diffusion pattern of these two fraction, Fig. 6. In that case, the two split ends from each of these fractions merged with two well resolved bands in the adjacent diffusion pattern of Fraction E1. Accordingly, it must be concluded that the split ends indicate different specificities of components of nearly identical diffusion rates and mobilities.

*Specificity Relationships by Immunoelectrophoresis*—The preceding observations of a major common specificity in chemically distinct fractions were confirmed conclusively by a novel adaptation of immunoelectrophoresis. Fractions E1 and D5 were selected for this demonstration because they represent the extremes in the method of separation. Moreover, Fraction E1 migrated toward the anode and Fraction D5 migrated toward the

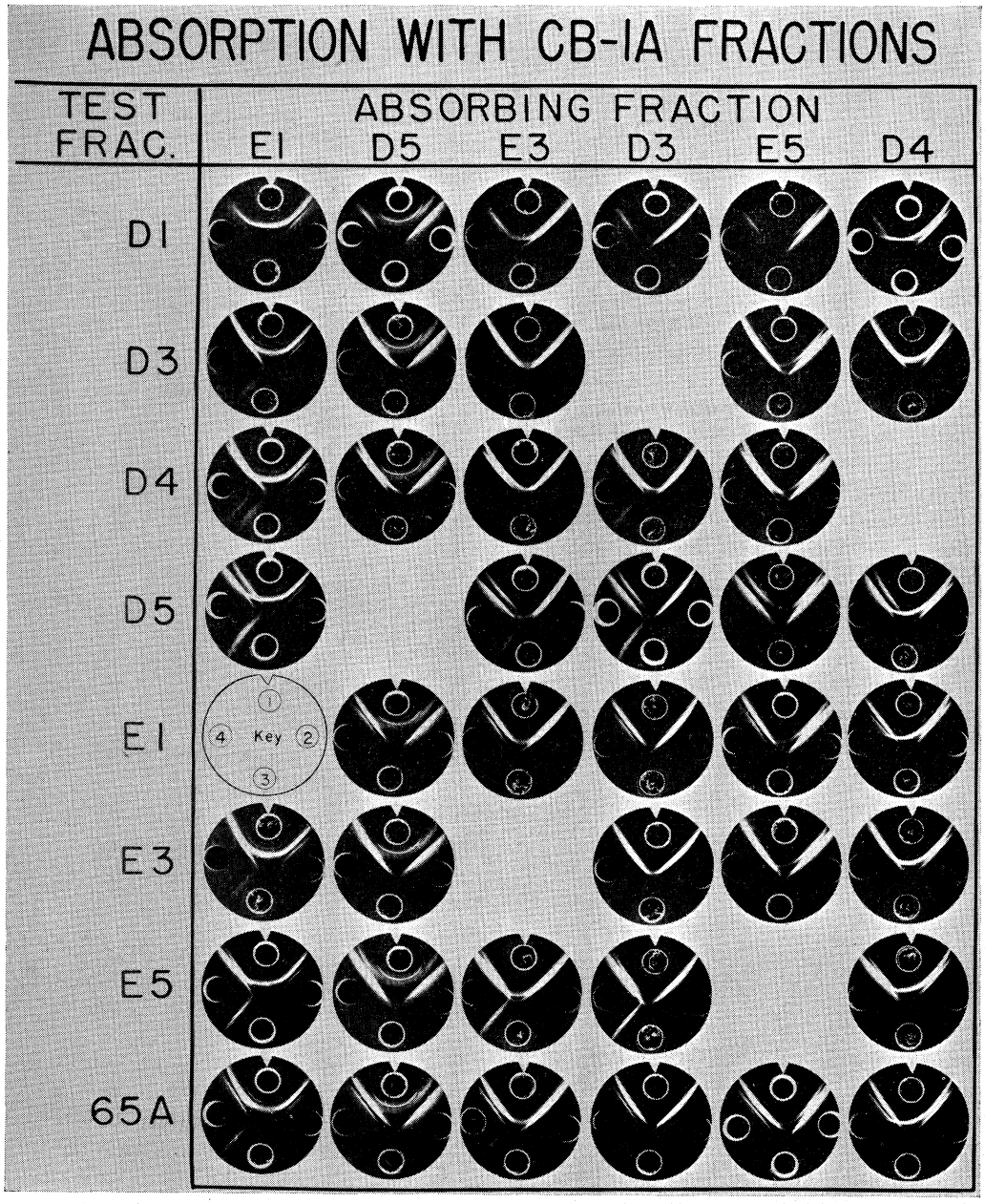


FIG. 8. Specificity relationships by absorption technique. Tests of antiserum absorbed with one fraction with the other fractions. Experimental details described in "Methods." Well 1, antiserum; Well 2, absorbing fraction; Well 3, absorbed antiserum; Well 4, test fraction. Interpretation of precipitate lines between wells: 1 and 2, absorbing fraction with antiserum (positive control); 2 and 3, absorbing fraction with absorbed antiserum (negative control); 3 and 4, test fraction with absorbed antiserum; 4 and 1, test fraction with unabsorbed antiserum (positive control); 1 and 3, excess antigen in absorbed antiserum with unabsorbed antiserum.

cathode in both the electrophoretograms (Fig. 3) and in the immunoelectrophoretograms (Fig. 9). The results are shown in Fig. 10.

In one half of an immunoelectrophoresis plate (*top plate* in Fig. 10), Fraction D5 was placed in the well nearer the anode and Fraction E1 in the well nearer the cathode. During the electrophoresis the fractions migrated toward each other, and during the immunodiffusion the ends of the principal lines of precipitate merged in a reaction of identity. In the *lower half* of this same plate the position of the fractions was reversed so that they migrated away from each other. The gap between the diffusion patterns confirms the chemical distinctness of Fractions E1 and D5. When the wells containing Fractions E1 and D5 were moved closer together as in the *lower half* of the *lower immunoelectrophoresis plate* of Fig. 10, the ends of the lines of precipitate of Fractions E1 and D5 joined even when the two fractions migrated away from each other.

*Specificity Relationships by Combined Immunoelectrophoresis and Gel Double Diffusion*—The antigenic similarity of the principal components of Fractions E1 and D5 were shown also by a combination of immunoelectrophoresis and gel double diffusion. Fractions E1 and D5 were placed in wells nearer the anode and cathode, respectively, and subjected to electrophoresis as above. After the electrophoresis, wells were formed in the gap area, and

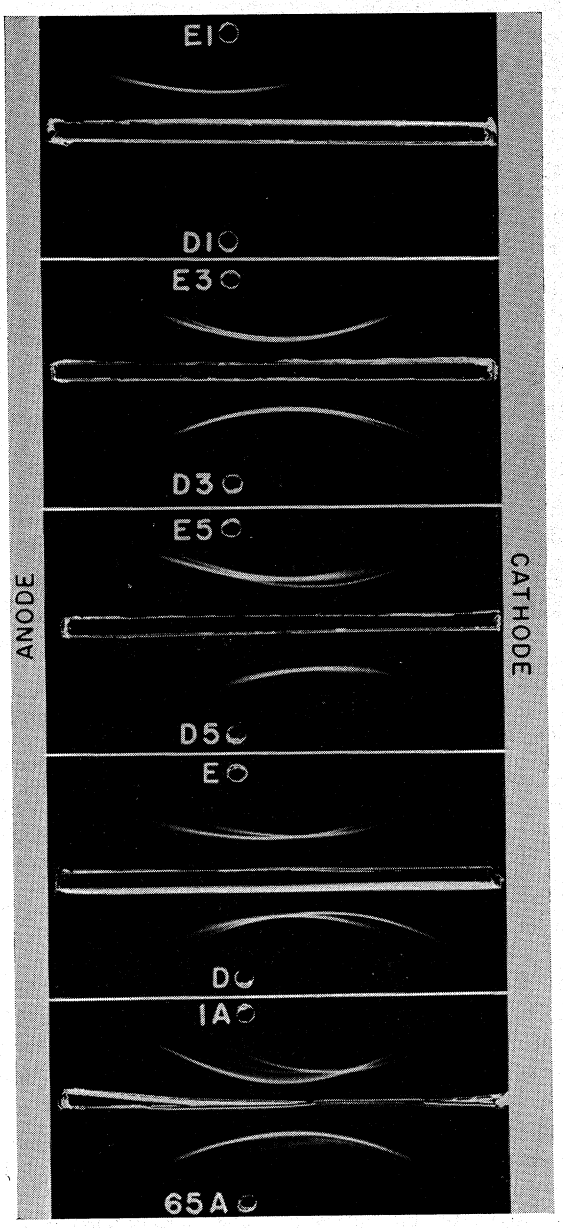


FIG. 9. Immunoelectrophoretic patterns of 1A and fractions of 1A. Agar, 0.8% in barbitone buffer, pH 8.6; depth, 4 mm; glass plate, 82- × -102 mm. Antigen holes, 3 mm in diameter, 45 mm from anode, and 15 mm from antiserum trough. Antiserum trough, 3 mm wide and 75 mm long. Fraction solutions, 27 μl, containing 0.5 mg of fraction nitrogen per ml in barbitone buffer. Electrophoresis, 4 hours at room temperature at constant voltage of 150 volts, current, 10 ma at start and 14 at end. After the electrophoresis, 1.0 ml of antiserum was added to the trough and immunodiffusion proceeded at 24 ± 1° for 4 days.

solutions of Fractions E1 and D5 were added to these wells as shown in Fig. 11. Antiserum was added to the troughs and the diffusion part of the immunoelectrophoresis was completed. The E1 and D5 solutions added after the electrophoresis closed the gap with joining lines of precipitate thus further confirming the similar specificities of Fractions E1 and D5.

## DISCUSSION

In view of the results described in this paper, demonstration of inherent antigenic or allergenic properties in chemically distinct components of Fraction 1A cannot be taken as evidence of distinct antigenic or allergenic specificities. This observation appears to be of more general applicability than to Fraction 1A. For example, an analogy to the specificity relationships of Fractions E1 and D5 has been described for Fraction CS-1A from cottonseed by another technique with the use of human reaginic serum (24). Fraction CS-1A, like Fraction 1A, is a complex mixture of proteins and polysaccharidic proteins. The principal components of Fraction CS-1A have a common allergenic specificity like that shown by Fraction 1A (24).

Two fractions of CS-1A, (CS-13)D1A and (CS-13)E5F6, represented the extremes of separation obtained by dialysis and ion exchange fractionation (7). That Fractions (CS-13)D1A and (CS-13)E5F6 were separated completely was shown by an ion exchange method of characterization (7). Also, that Fraction (CS-13)D1A did not precipitate Fraction CS-13E rabbit antiserum in gel double diffusion up to 200 μg of fraction nitrogen per ml, whereas the threshold concentration for precipitation of Fraction (CS-13)E5F6 was only 0.17 μg of fraction nitrogen per ml confirmed the distinctiveness of the two fractions. Nevertheless, Fraction (CS-13)D1A was twice as effective as Fraction (CS-13)E5F6 in inciting reactions and one-half as effective in neutralizing reagins (this range is the same as the experimental error of the method) in sites passively sensitized with human reaginic serum (Table III of Reference 24). Fraction (CS-13)-E5F6 neutralized the passively sensitized sites to further test with Fraction (CS-13)D1A, but Fraction (CS-13)D1A did not neutralize the sites to subsequent test with Fraction (CS-13)-E5F6. However, Fraction (CS-13)D1A completely inhibited the precipitin reaction of Fraction (CS-13)E5F6.[4] These results showed by other techniques that components of Fraction CS-1A, which are chemically distinct, have common antigenic and allergenic specificity relationships like those of Fraction CB-1A described in this paper.

With use of the Schultz-Dale technique, Layton, Moss, and DeEds (12) reported the demonstration of six distinct antigenic specificities in fractions of 1A obtained by separation on a DEAE-cellulose column. This conclusion was based on their observation that uterine muscle from guinea pigs sensitized to Fraction 1A reacted to the fractions represented by peaks in the chromatogram. The fractions were tested in sequence in the order of their elution from the column. Initial reaction and desensitization to antecedent fractions did not desensitize the muscle to reaction with subsequent fractions. Tests for cross-reactions between individual fractions were not reported. In view of the results of the Schultz-Dale experiments reported in the present paper, the subsequent uterine contractions elicited by later fractions observed by Layton *et al.* (12) could have resulted from an increased concentration, or potency, of antigen with the same specificity as contained in the antecedent fractions, in accord with the law of multiple proportions (9, 25, 26). Accordingly, antigens of different chemical structure but with a common specificity could have been present in all of the fractions and fail to be detected by the procedure used. In a subsequent publication Layton *et al.* (13) reported that serial chromatographic fractions appeared to be contaminated by material trailing from the preceding peaks, and concluded that the evidence only demonstrated the presence of more than one sensitizing antigen in Fraction 1A.

[4] Unpublished data.

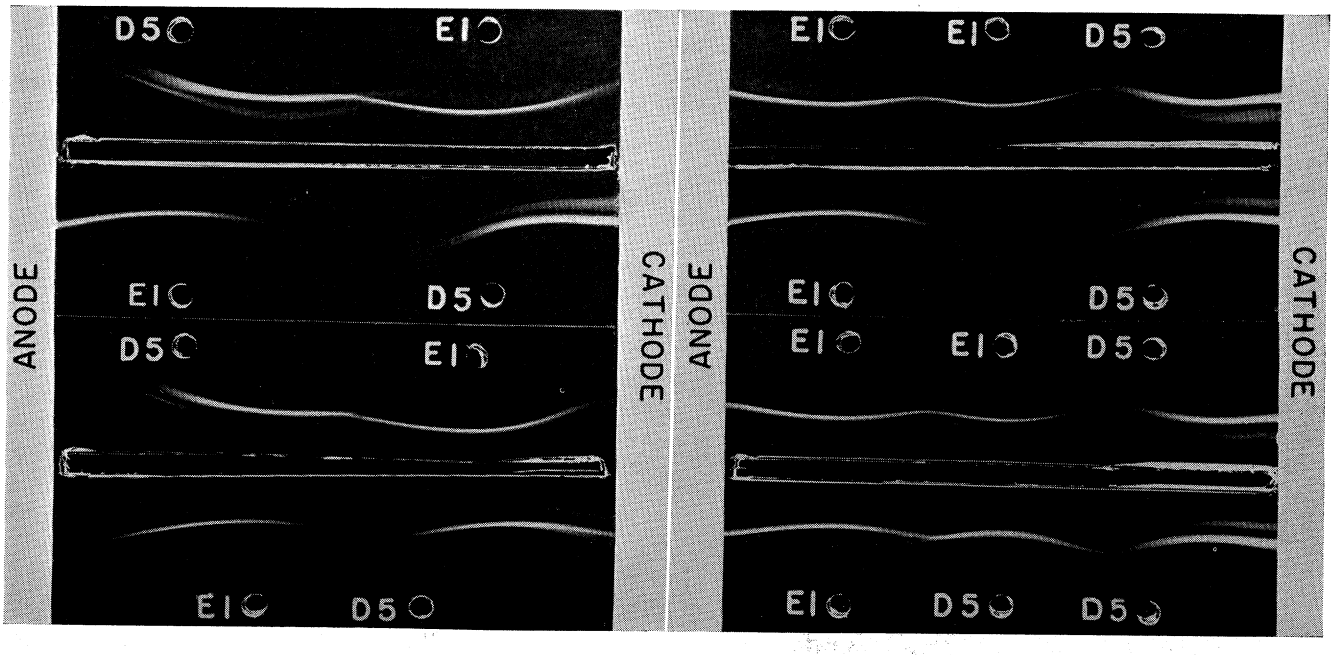


FIG. 10. Immunoelectrophoresis of Fractions E1 and D5. *Upper half of top plate* shows relatively greater migration of Fraction D5 toward cathode and of Fraction E1 toward anode so that main lines of precipitate join. *Lower half of top plate* similar to *upper half* except that positions of Fractions E1 and D5 were reversed so that because of differences in their migration the lines of precipitate do not join. *Upper half of lower plate* similar to *upper half of top plate*. *Lower half of lower plate* similar to *lower half of top plate* except that Fractions D5 and E1 wells were 20 mm instead of 40 mm apart. General conditions of the immunoelectrophoresis similar to those described in Fig. 9. Antigen wells 30 mm from electrodes and 40 mm between wells. Diffusion proceeded for 4 days at 24 ± 1°.

FIG. 11. Combination of immunoelectrophoresis and gel double diffusion of Fractions D5 and E1. General conditions of immunoelectrophoresis similar to those of Fig. 9. The immunoelectrophoresis setup of both plates is similar to that of the *lower half of the upper plate* of Fig. 10. After the electrophoresis, the central wells were punched midway (20 mm) between the other two wells 27 μl of the indicated fraction solution containing 0.25 mg of fraction nitrogen per ml then was added to the central wells. Then 1.0 ml of antiserum was added to the trough and diffusion proceeded for 4 days at 24 ± 1°.

Layton *et al.* (13, 27) also showed that Fractions 1A and CB-1A SRI could be resolved into six or more components by paper strip electrophoresis. Each major component band was shown to contain antigenic material by passive cutaneous anaphylaxis in guinea pigs that were sensitized with rabbit antiserum to crude castor bean protein. The individual bands were also allergenic in humans (13). Layton *et al.* interpreted these observations to indicate that the separable components of Fraction 1A contained several antigens and allergens with individually distinguishable specificities. However, experiments designed to detect cross-reactions by direct comparison of one fraction with another were not reported. Evidence presented in the present paper shows that the major components of the separable fractions of 1A mutually cross-react with each other. This indicates that the major antigenic components of the several fractions exhibit a common antigenic specificity.

It is evident that Fractions D5 and E5 contain a minor common specificity distinct from the major common specificity as shown in gel double diffusion, immunoelectrophoresis and Schultz-Dale tests. Other minor specificities are possible, but their relative proportions are so low that they cannot be distinguished with certainty. From available evidence the possibility of artifacts cannot be excluded definitely, especially in the gel double diffusion tests. The significance of minor specificities with regard to allergenic properties of Fraction 1A must await further study. Obviously, reagins specific for these components could occur.

The carbohydrate-free allergenic fraction, 65A, previously was shown to be a mixture by solubility determination (6). By analogy to the corresponding carbohydrate-free allergen, CS-60C from cottonseed (3), it was suggested that Fraction 65A was a mixture of active, closely related proteins. By making use of a paper electrophoretic fractionation of 65A, Layton *et al.* (28) reported the presence of five bands all of which gave positive cutaneous reactions on the skin of castor bean sensitive persons. Evidence regarding the specificity relationships of the fractions was not given by Layton *et al.* The electrophoretogram, the immunoelectrophoretogram, and gel double diffusion of Fraction 65A indicated three bands or groups of components (a fourth faint band was visible on the original electrophoretogram). Decision cannot be made regarding the specificity relationships of these components of Fraction 65A from the available evidence. However, Fraction D5 completely absorbed antibodies specific for Fraction 65A even though the electrophoretograms showed that the two heaviest bands of Fraction 65A did not overlap any bands of Fraction D5. This observation indicated the possibility that different proteins of Fraction 65A may have similar specificities.

The concept that Fractions 1A and CS-1A (and the other natural proteoses) contain a multiplicity of components that exhibit similar allergenic and antigenic specificities has been developed in studies reported during the past 22 years (1–7, 9, 10, 24). This concept is based on two factors: (*a*) active protein or proteins combined with various proportions of polysaccharidic carbohydrate, in which combination protein is the allergenic and antigenic determinant, and (*b*) structural difference in the protein

moiety too slight to impart distinct specificities (3). Thus, for example, the chemical differences that permitted separation of Fractions E1 and D5 might be attributed to different carbohydrate contents of the polysaccharidic-protein complex, whereas, separation of Fraction D4 from Fraction D5 could be due to differences in the proteins *per se*.

Pertinent to Postulate *b* is the explanation by Grabar (29) of the long curved arcs in the immunoelectrophoretic patterns of γ-globulins. Grabar believes that these are caused by a series of substances of different mobilities but of the same antigenic specificity. The immunoelectrophoretic patterns of the 1A fractions are strikingly similar to those of the serum γ-globulins.

Berrens and Young (30) have reported that the allergenic fraction of ipecac is a mixture of similar glycoproteins whose differences are due to variation in the carbohydrate binding. They believe that the antigenic determinant structure is located in the protein which is identical for all of the molecular species of the mixture.

The status of our present concept of Fraction 1A is summarized below because of the complexity of the castor bean allergen problem and because our views differ from those of other authors.

Fraction CB-1A consists of a complex mixture of low molecular weight proteins, and proteins combined with varying proportions of polysaccharidic carbohydrates (1, 6, 15). The components of Fraction 1A are immunologically distinct from other antigens of the seed (10, 15). A major common or identical specificity is found in all of the subfractions of Fraction 1A even though they are completely separated from each other, and thus chemically distinct. Fraction 1A is antigenically heterogeneous (8) and contains one and possibly two or more minor specificities.

With reference to the allergenic activity of Fraction 1A, it is possible that each distinct antigen may function also as a distinct allergen. However, the evidence observed, so far, favors the view that the major antigenic specificity that pervades all subfractions of Fraction 1A, is also the principal allergen. At least one other castor bean allergen, distinct from Fraction 1A, has been observed in the meal (Table VI of Reference 1).

In our experience, clinical sensitivity to castor beans has been associated with cutaneous sensitivity to Fraction 1A and with the presence of reagins specific for Fraction 1A demonstrable by passive transfer. The threshold concentration of Fraction 1A required for cutaneous reactions varies with individual sensitivity, but is approximately 1:2,000,000. Fraction 1A, 1 mμg, will elicit response in skin sites passively sensitized with 0.05 ml of a potent castor bean reaginic serum (18). Fraction 1A is nontoxic (15, 18, 31). Fraction 1A has induced formation of blocking antibodies in man (31) and in one case, administration of Fraction 1A under experimental conditions induced transitory formation of reagins and cutaneous sensitivity in man (31).

Fraction 1A is composed of amino acids and is characterized by the absence of tryptophan and the presence of relatively high proportions of arginine and glutamic acid (1). Fraction 1A is extremely stable to heating even in alkaline solution (32). The 1A content of 10 varieties of decorticated, defatted castor beans ranged from 6 to 9% and that of six samples of castor pomace from foreign and domestic sources ranged from 0.092 to 4.2% (8).

SUMMARY

An ion exchange fractionation of castor bean allergen, CB-1A, has been made, and the fractions were characterized by cellulose acetate electrophoresis. The antigenic specificity relationships of the fractions have been studied by the Schultz-Dale technique, by gel double diffusion, by immunoelectrophoresis, by a combination of immunoelectrophoresis and gel double diffusion, and by absorption techniques. Two chemically distinct components of Fraction CB-1A, E1 and D5, were shown to have similar antigenic specificities by a combination of immunoelectrophoresis and gel double diffusion. The previously recognized concept that Fraction CB-1A is a complex mixture of polysaccharidic proteins and proteins having a major common antigenic specificity has been confirmed and extended. At least one and possibly two antigenic specificities distinct from the major common specificity were observed. Our current concept of the status of Fraction CB-1A in relationship to castor bean allergenicity has been summarized.

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